PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	James A. Williams	Group No.:	1816
Serial No.:	08/704,159	Examiner:	Evelyn Rabin
Filed:	08/28/1996		
Entitled:	MULTIVALENT VACCINE FOR *CLOSTRIDIUM BOTULINUM* NEUROTOXIN		

DECLARATION OF JAMES A. WILLIAMS UNDER 37 CFR §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: 4/15/99 By: Anne M. Neiswander

Sir:

1. I, James A. Williams, am sole inventor of the subject matter embodied in the above-identified patent application.

2. I am the subject of the attached Curriculum Vitae and author of the publications shown on the list attached thereto. On the basis of the information and facts contained in these documents, I submit that I am an expert in the fields of molecular biology, biochemistry and immunology and am qualified to speak on the level of ordinary skill in these fields.

3. I have read and understand the above-identified patent application, the Office Action from the Patent Office mailed October 15, 1998, and the references which were cited by the Examiner in the office action and which were authored by Thompson *et al.*, Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel.

4. The work comprising producing the claimed vaccines which is described in the specification, which is the subject of Claims 10-14 and 25-28 as amended in the

General of the Department of the Army to the Federal Food and Drug Administration], which is **six years** prior to the filing date (*i.e.*, August 28, 1996) of the instant application.

Furthermore, as disclosed by the specification, I have successfully used the art-accepted mouse neutralization assay to determine the biological activity of **portions** of both *Clostridium botulinum* type B (*i.e.*, the C fragment of *Clostridium botulinum* type B[3]) and type E (*i.e.*, the C fragment of *Clostridium botulinum* type E[4]) toxins.

Based on the above-described experimental results, and on my knowledge of the relevant art, it is my opinion that the ability of a fusion protein which contains a non-toxin protein and **any portion** of *Clostridium botulinum* type B and/or type E toxins, to generate neutralizing antibodies may be determined using the teachings of the specification in combination with routine steps of the art-accepted mouse neutralization assay, and that this assay is well within the ordinary skill in the art.

8. The Examiner argued that the term "at least a portion of" is indefinite because "the artisan would not necessarily know which portions would result in neutralizing antibodies, so the portions would not be understood by the artisan."[5]

9. The specification defines the term "portion" when in reference to a protein as a fragment which "may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid."[6] Based on this definition, and my understanding of it as one of skill in the art, it is my opinion that the term "at least a portion of" a toxin refers to **fragments which range in size from 4 amino acids to the entire amino acid sequence of the toxin.**

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3 A fusion protein (HistBotB) containing a polyhistidine tag and the C fragment of *Clostridium botulinum* type B toxin was made as described in Example 35 (specification, pages 227-229), and neutralizing antibodies to this fusion protein were generated as described in Example 36 (specification, pages 229-230).

4 A fusion protein (HistBotE) containing a polyhistidine tag and the C fragment of *Clostridium botulinum* type E toxin was made as described in Example 41 (specification, pages 244-246), and neutralizing antibodies to this fusion protein were generated as described in Example 42 (specification, pages 247-248).

5 Office Action, paragraph bridging pages 3 and 4.

6 Specification, page 19, lines 3-6.

10. The Examiner argued that Claims 10-14 and 25-28 were obvious under 35 U.S.C. § 103(a) over Thompson *et al.* in view of Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel.

11. The **solubility** of fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins was **unexpected** based on the prior art's teaching. LaPenotier *et al.* [LaPenotier *et al.* (1993) "Development of a Molecular Engineered Vaccine for *C. Botulinum* Neurotoxins, in *Botulinum and tetanus Neurotoxins*, B.R. DasGupta (Ed.), Plenum Press, New York, p. 463-466] disclosed a fusion protein of *Clostridium botulinum* type A toxin C fragment and maltose binding protein (MBP). LaPenotier *et al.* also disclosed that its fusion protein formed inclusion bodies (*i.e.*, was **insoluble**). The insolubility of LaPenotier *et al.*'s *Clostridium botulinum* type A toxin fusion protein suggested that other fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins might also be insoluble. Based on the disclosure of LaPenotier *et al.*, it was surprising to find that I was able to successfully produce a **soluble** *Clostridium botulinum* type B toxin/polyhistidine fusion protein and a *Clostridium botulinum* type E toxin/polyhistidine fusion protein.

12. The **solubility** of fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins was **unexpected** based on my own experimental work with two toxins (type A and type B toxins) derived from *Clostridium botulinum*.

With respect to *Clostridium botulinum* type A toxin, I confirmed the **insolubility** of LaPenotier's *Clostridium botulinum* type A toxin C fragment/MBP fusion protein by observing that (a) most (*i.e.*, about 90%) of this protein was removed by centrifugation, (b) long term-storage for more than 2 weeks resulted in precipitation of most of this affinity-purified fusion protein, and (c) the majority (*i.e.*, 75%) of this fusion protein persisted in pellets even after sonication of these pellets. The insolubility of the *Clostridium botulinum* C fragment/MBP fusion protein which was disclosed in Example 24 of the specification is consistent with LaPenotier's statements that this fusion protein formed insoluble inclusion bodies. The results of my work which demonstrated the insolubility of LaPenotier *et al.*'s *Clostridium botulinum* type A toxin fusion protein suggested that other fusion proteins

containing at least a portion of *Clostridium botulinum* type B and/or type E toxins would also be insoluble. I empirically determined the surprising solubility of the *Clostridium botulinum* C fragment type A toxin/polyhistidine fusion protein by using, among other things, a relatively weak promoter to drive expression of the fusion protein.

My initial experimental observations also demonstrated the **expected insolubility** of fusion proteins which contained portions of *Clostridium botulinum* type B toxin. I initially prepared four expression vectors [pHisBotB kan lacIq T7lac, pHisBotB kan T7lac, pHisBotB kan lacIq T7, and pHisBotB amp T7lac] which encoded a fusion protein containing *Clostridium botulinum* type B toxin C fragment and a polyhistidine tag. Fermentation of the B121(DE3) strain of *Escherichia coli* containing each of the **four** expression vectors yielded only **insoluble** fusion protein.[7] This demonstrated that, unlike the *Clostridium botulinum* type A toxin which I was able to express in a soluble form by using a relatively weak promoter, the *Clostridium botulinum* type B toxin remained insoluble even when I used a similar approach (*i.e.*, a relatively weak promoter) to drive expression of the fusion protein containing it. Based on the teachings of LaPenotier *et al.* and my initial failure to express soluble fusion proteins containing a portion of either type A or type B toxins, it was **surprising** to later find that I was able to successfully produce a **soluble** *Clostridium botulinum* type B toxin C fragment/polyhistidine fusion protein. This success was empirically arrived at through the simultaneous overexpression of three of the initially-used four expression vectors together with folding chaperones as demonstrated in the specification using the pHisBotBkan lacIq T7lac/pACYCGro, pHisBotB kan T7lac/pACYCGro and pHisBotBkan lacIq T7/ pACYCGro vectors.[8] I empirically determined the surprising solubility of the *Clostridium botulinum* type B toxin C fragment/polyhistidine fusion protein. Nothing in Thompson *et al.*, Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel (alone or in combination) either suggests or teaches this surprising solubility of the *Clostridium botulinum* type B toxin C fragment/polyhistidine fusion protein.

With respect to *Clostridium botulinum* type E toxin, based on the teachings of LaPenotier *et al.* and on my above-discussed initial failure to express soluble fusion proteins

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7 Specification, Example 38, pages 232-234.

8 Specification, Example 39, pages 235-238.

containing either *Clostridium botulinum* type A toxin C fragment/polyhistidine fusion protein or *Clostridium botulinum* type B toxin C fragment/polyhistidine fusion protein, I **could not be sure** that I would produce a soluble fusion protein containing *Clostridium botulinum* type E toxin portions and a non-toxin protein. Thus, I was surprised to find that I was able to successfully produce a **soluble** *Clostridium botulinum* type E toxin C fragment/polyhistidine fusion protein even without need to resort to the approaches (*e.g.*, use of a relatively weak promoter as used for type A toxin, and use of folding chaperones as used for type B toxin), which I empirically determined for the other *Clostridium botulinum* two toxin types. For example, fermentation of *Escherichia coli* B121(DE3) strain containing each of three initially-prepared expression vectors [pHisBotE kan lacIq T7lac, pHisBotE kan T7, and pHisBotE kan lacIqT7] which encoded a fusion protein containing *Clostridium botulinum* type E toxin C fragment and a polyhistidine tag resulted in expression of **soluble** histidine-tagged proteins of the predicted molecular weight for the type E toxin.[9] I empirically determined the surprising solubility of the *Clostridium botulinum* type E toxin C fragment/polyhistidine fusion protein. Nothing in Thompson *et al.*, Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel (alone or in combination) either suggests or teaches this surprising solubility of the *Clostridium botulinum* type E toxin/polyhistidine fusion protein.

13. The ability of fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins to **generate neutralizing antibodies** was **unexpected** based on the teachings of Kleid. Kleid (which was cited by the Examiner) teaches that it is "not known" whether fusion proteins may successfully be used for generating neutralizing antibodies in view of "[s]ome problems that could be expected to occur," including, (a) "that the immunogenic site may not be properly exposed," (b) "the peptide sequences(s) within that site may not be able to form into the correct configuration," and (c) "the immunogenic site may require disulfide bonding to bring two distant parts of a protein or two different peptide chains into close proximity to form an antigenic site."[10]

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9 Specification, Example 44, pages 250-251.

10 Kleid, page 29, "Summary" section.

14. The ability of fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins to **generate neutralizing antibodies** was **unexpected** based on the prior art's teaching. The art disclosed that expression of the *Escherichia coli* verotoxin 2 cell binding domain in *Escherichia coli* cells resulted in the production of antibodies that are **not neutralizing** [Acheson et al. (1995) "Expression and purification of Higa-like toxin II B subunits," Inf. Immunity 63:301-308]. This suggested that while antibodies may be generated against a toxin protein (*e.g.*, the *Escherichia coli* verotoxin 2 cell binding domain), these antibodies are not necessarily neutralizing.

15. The ability of fusion proteins containing at least a portion of *Clostridium botulinum* type B and/or type E toxins to **generate neutralizing antibodies** was **unexpected** based on my following experimental work which attempted to determine whether neutralizing antibodies were generated against toxins derived from two organisms.

A. Antibodies raised against *Staphylococcus aureus* enterotoxin B proteins are not neutralizing

I expressed the entire *Staphylococcus aureus* enterotoxin B protein in two overlapping fragments. One fragment contained approximately two-thirds of the N-terminal region, while the other fragment contained two-thirds of the C-terminal region of the enterotoxin. Both fragments were expressed as **insoluble** inclusion bodies. The inclusion bodies were solubilized and used to immunize chickens. **Immunization resulted in generation of high titers of antibodies to each protein as assayed by ELISA and Western blot hybridization. The mouse neutralization assay was used to determine the neutralization activity of these** antibodies against the entire native *Staphylococcus aureus* enterotoxin B protein. In this assay, antibodies against the two solubilized *Staphylococcus aureus* enterotoxin B fragments showed **no neutralizing activity**. These results suggested that while a solubilized insoluble toxin may generate antibodies, these antibodies are not necessarily neutralizing.

B. Antibodies raised against *Clostridium difficile* toxin A fragments are not neutralizing

Expression of the entire (80 Kd) *Clostridium difficile* toxin A cell binding domain in *Escherichia coli* resulted in an **insoluble** protein. While this protein resulted in the production

of high titer antibodies in chickens (as measured by ELISA and Western blot hybridization), these antibodies **failed to neutralize** the toxicity of *Clostridium difficile* toxin A in a hamster toxicity model.

Since the *Clostridium difficile* binding domain contains more than 30 multiple repeats of a cell binding domain, I hypothesized that expression of this binding domain in two fragments, each containing multiple copies of the repeats, may generate neutralizing antibodies if the expressed fragments were soluble. However, while expression of the *Clostridium difficile* cell binding domain in two 40 Kd fragments resulted in two soluble fragments, these soluble proteins were **unable** to bind to neutralizing antibodies in a hamster toxicity model.

These results demonstrated that regardless of whether a toxin is expressed as an insoluble or soluble protein which elicits antibodies, these antibodies are not necessarily neutralizing.

16. It is my opinion, based on the expected problems associated with generating fusion proteins which are capable of generating neutralizing antibodies as warned against by Kleid (paragraph 13, *supra*), failure of the prior art to generate neutralizing antibodies as disclosed by Acheson *et al.* (paragraph 14, *supra*), as suggested by my own experimental work (paragraph 15, *supra*), and on my knowledge of the art, that at the time the invention was filed (*i.e.*, August 28, 1996) one of ordinary skill in the art would not have expected to generate fusion proteins which contained a non-toxin sequence and at least a portion of *Clostridium botulinum* type B and/or type E toxins and which were capable of eliciting neutralizing antibodies. **I empirically determined the ability of each of a *Clostridium botulinum* type B toxin C fragment/polyhistidine fusion protein (HisBotB)[11] and of type E toxin C fragment/poly-histidine fusion protein (HisBotE)[12] to generate neutralizing antibodies.** Nothing in Thompson *et al.*, Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel (alone or in

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11 Specification, Example 36, pages 229-230.

12 Specification, Example 42, pages 247-248.

combination) either suggests or teaches this unexpected ability of the HisBotB and HisBotB fusion proteins to generate neutralizing antibodies.

17. The claimed fusion proteins which comprise a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type B and/or type B toxins have qualitatively unexpected properties (including solubility and ability to generate neutralizing antibodies) compared to the prior art. These unexpected properties of the claimed invention are not suggested by Thompson *et al.*, Binz *et al.*, Roitt, LeClerc *et al.*, Kleid, and Siegel either alone or in combination. I arrived at the unexpected properties of the claimed fusion proteins empirically, rather than on the basis of what the prior art discloses.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: 4/12/99 Signed: James A. Williams
JAMES A. WILLIAMS

CURRICULUM VITAE

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BioNebraska Pharmaceuticals, Inc.
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Education:

B. S., Biology with Honors	University of Victoria, Canada, 1981
Ph.D., Genetics	University of Alberta, Canada, 1988

Professional Experience:

BioNebraska, Inc — **11/96 - Present**

<u>Vice President and Director, Molecular Biology &Process Development</u>, BioNebraska, Lincoln. (1998-present)
<u>Vice President and Director, Molecular Biology</u>, BioNebraska, Lincoln. (1997-present)
<u>Director, Molecular Biology</u>, BioNebraska, Lincoln. (1996 - present):

- Project coordinator for Glucagon-like Peptide manufacturing program.
- Director, Molecular Biology, Genetics, Microbiology and Process Development groups.
- Development of recombinant peptide expression technologies.
- Responsible for cGMP manufacture and QC testing of cell banks and peptide pharmaceuticals.

Ophidian Pharmaceuticals, Inc — **10/93 -11/96**

<u>Section Manager</u>, Ophidian Pharmaceutical, Madison. (95-96)
<u>Senior Scientist</u>, Ophidian Pharmaceutical, Madison. (93-94)
<u>Consultant</u>, Ophidian Pharmaceutical, Madison. (93)

- Director, New Technology Discovery group..
- Process Definition of multiple recombinant antigen expression and purification technologies.
- Process Development of recombinant protein fermentation and purification methodologies.
- Developed QC analytical methods for recombinant protein characterization and release testing.

Steritech Pharmaceuticals, INC — **1992**

<u>Consultant</u>, Steritech Pharmaceutical, San Francisco.

- Cloned and expressed recombinant antigens in *E. coli* for diagnostic assays.

Postdoctoral Research — **1989-1993**

<u>University of Wisconsin</u>, Madison with Dr. Sean Carroll. (10/90 - 10/93)
<u>University of British Columbia</u>, with Dr. Tom Grigliatti. (7/89 - 7/90)

- Drosophila research. Multiple Molecular Biology, Biochemistry, Genetic and Immunology projects.

Technical Expertise:

Molecular biology, protein over expression and fermentation

- DNA manipulations including cloning and manipulation of M13, plasmid and lambda phage vectors, DNA isolation. Southern hybridization analysis, site directed mutagenesis.
- RNA manipulations including the isolation of RNA, and Northern hybridization analysis.
- Genomic library construction and chromosome walking.
- cDNA library construction and screening.
- cDNA library construction from RNA viruses and cloning of viral genomes.
- DNA sequencing methodologies.
- PCR amplification methodologies.
- Recombinant antigen optimization utilizing protein evolution methodologies.
- Expression vector design, and recombinant antigen expression in *E. coli*.
- 15 liter scale fermentation of E. coli (BioFlo IV, New Brunswick Scientific) and fermentation media and process development.
- Methodologies for processing fermentation cultures (e.g. continuous flow centrifugation, high pressure homogenization, flocculation).

Protein purification and characterization

- Chromatographic purification of proteins (e.g. sizing, affinity, reverse phase, ion exchange chromatography).
- Biochemical analysis of proteins (HPLC, isoelectric focusing, native and SDS-PAGE).
- Protein formulation and stability studies.
- Polyclonal antibody generation.
- Western analysis.
- ELISA or bead agglutination assay development.
- Protein modification (fluorescence, biotin or enzyme labeling of proteins).

Expression vector design

- Construction of proprietary expression vectors and recombinant antigen purification strategies.
- Construction of Production E. coli host utilizing gene disruption/replacement methodologies.

Other

- Immunohistochemistry.
- Immunological screening of expression libraries.
- Protein/protein interaction screens.
- Immunological assay development (e.g. antibody evaluation in complement or phagocytotic killing assays).
- Tissue culture propagation of mammalian cell lines.
- Fluorescence polarization and quenching assays.
- Animal models of infection disease.
- Random peptide library screening.

Regulatory and manufacturing

- Working familiarity with FDA regulations for biological products.
- cGMP peptide pharmaceutical manufacturing experience.
- Development of immunological, microbiological, HPLC and molecular biology Quality Control testing procedures in support of peptide pharmaceutical manufacturing program.
- Drafting of SOP's, specifications and manufacturing batch records.
- Cleanroom setup and establishment of environmental monitoring program.

Publication Record:

Refereed Publications:

Kink, J. A. and **Williams, J. A.** (1998). Antibodies to recombinant *Clostridium difficile* Toxins A and B are an effective treatment and prevent relapse of *C. difficile*- associated disease in a hamster model of infection. *Infection and Immunity* 66: 2018-2025.

Williams, J. A., Paddock, S. W., Vorwerk, K., and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* 368: 299-305.

Carroll, S. B., Gates, J., Keys, D. N., Paddock, S. W., Panganiban, G., Selegue, J. E., and **Williams, J. A.** (1994). Pattern formation and eyespot determination in butterfly wings. *Science*, 265: 109-114.

Williams, J. A., Paddock, S. W. and Carroll, S. B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. *Development* 117: 571-584.

Bazin, C., **Williams, J.**, Bell, J. and Silber, J. A. (1993). A deleted *hobo* element is involved in the unstable thermosensitive vg^{al} mutation at the vestigial locus in *Drosophila melanogaster*. *Genetical Res.* 61: 171-177.

Clegg, N. J., Whitehead, I. P., **Williams, J. A.**, Spiegelman, G. B. and Grigliatti, T. A. (1993). A developmental and molecular analysis of *Cdc2* mutations in *Drosophila melanogaster*. *Genome* 36: 676-685.

Heslip, T. R., **Williams, J. A.**, Bell, J. B. and Hodgetts, R. B. (1992). A *P* element chimera containing captured genomic sequences was recovered at the *vestigial* locus in *Drosophila* following targeted transposition. *Genetics* 131: 917-927.

Williams, J. A., Bell, J. B. and Carroll, S. B. (1991). Control of *Drosophila* wing and haltere development by the nuclear vestigial gene product. *Genes Devel.* 5: 2481-2495.

Williams, J. A., Scott, I. M., Atkin, A. L., Brook, W. J., Russell, M. A. and Bell, J. B. (1990). Genetic and molecular analysis of vg^U and vg^W: two dominant *vg* alleles associated with gene fusions in *Drosophila*. *Genetics* 125: 833-844.

Williams, J. A., Atkin, A. L. and Bell, J. B. (1990). The functional organization of the *vestigial* locus in *Drosophila melanogaster*. *Mol. Gen. Genet.* 221: 8-16.

Williams, J. A. (1988). A molecular analysis of the *vestigial* locus in *Drosophila melanogaster*. Ph.D thesis, University of Alberta, Edmonton, Alberta.

Williams, J. A., Pappu, S. S. and Bell, J. B. (1988). Suppressible *P*-element alleles of the *vestigial* locus in *Drosophila melanogaster*. *Mol. Gen. Genet.* 212: 370-374.

Williams, J. A., Pappu, S. S. and Bell, J. B. (1988). Molecular analysis of hybrid dysgenesis-induced derivatives of a *P*-element allele at the *vg* locus. *Mol. Cell. Biol.* 8: 1489-1497.

Williams, J. A. and Bell, J. B. (1988). Molecular organization of the *vestigial* region in *Drosophila melanogaster*. *EMBO* 7: 1355-1363.

Molnar, C. M., Reece, T., **Williams, J. A.** and Bell, J. B. (1988). Transformation of *Drosophila melanogaster* with a suppressor tRNA gene (Sup3e $tRNA^{Ser}$) from *Schizosaccharomyces pombe*. *Genome* 30: 211-217.

Book Chapters and Review Articles:

Williams, J. A., Langeland, J. A., Thalley, B., Skeath, J. B. and Carroll, S. B. (1995). Production and

purification of polyclonal antibodies against proteins expressed in *E. coli.* DNA Cloning: Expression Systems, IRL Press.

Williams, J. A. and Carroll, S. B. (1993). The origin, patterning and evolution of insect appendages. *Bioessays* 15: 567-577.

Patents:

Williams, J. A., Kink, J. A., Clemens, C. and Carroll, S. B. *Clostridium difficile* toxin disease therapy. U.S. Patent 5,762,934 issued 6/98.

Williams, J. A., Kink, J. A., Clemens, C. and Carroll, S. B. Avain antitoxins to *Clostridium difficile* toxin A. U.S. Patent 5,601,823 issued 2/97.

An additional 8 patent applications are pending at the US Patent office.